

# ELECTROSTATIC BONDING TYPE MACROMOLECULAR MICELLE DRUG CARRIER AND DRUG CARRIED THEREON

[0001] This is a continuation of Serial No. 09/729,216, filed December 5, 2000, now abandoned, which is a continuation of Serial No. 08/584,329, filed January 11, 1996, now abandoned.

## FIELD OF THE INVENTION

[0002] The present invention relates to an electrostatic bonding macromolecular micelle drug carrier and drugs carried thereon. More particularly, the present invention relates to a novel macromolecular micelle drug carrier of a chargeable drug such as protein and DNA, which is useful in areas such as a drug delivery system (DDS) which carries a drug to a permissive site *in vivo* and causes the drug to stably display the functions and effects thereof, drugs to be carried by such a carrier, and a method of carrying a drug on this carrier.

## PRIOR ART AND PROBLEMS

[0003] Macromolecular micelle type drugs are attracting the general attention as a useful method for a drug delivery system (DDS), for example, and the present inventors have already proposed a macromolecular micelle type drug which causes physical adsorption of a hydrophobic drug by a block copolymer comprising a hydrophilic segment and a hydrophobic segment.

[0004] The macromolecular micelle type drug based on this physical adsorption is attracting general attention because of a new structure and the possibility of using same in practice.

[0005] According to studies carried out by the present inventors, however, it is now clear that there still remain problems to be solved. More specifically, the macromolecular micelle drug based on this physical adsorption, although being very excellent as a means to administer a hydrophobic drug, has a structure essentially characterized by physical

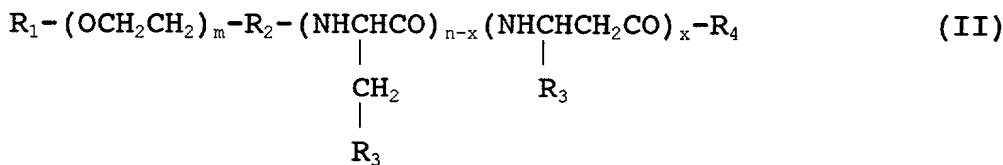
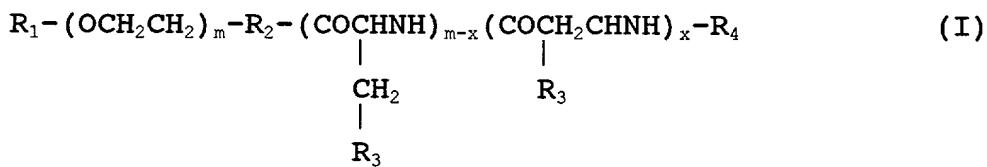
adsorption of a hydrophobic drug by a block copolymer. There has therefore been a drawback that the method has been applicable only to drugs having a sufficient hydrophobicity.

[0006] Under such circumstances, there is a demand for the achievement of a novel technical means applicable in a wide range, which permits stable carrying of a drug irrespective of whether the drug is hydrophobic or hydrophilic.

## SUMMARY OF THE INVENTION

[0007] The present invention provides an electrostatic bonding macromolecular micelle drug carrier comprising a block copolymer having a non-chargeable segment and a chargeable segment, which solves the above-mentioned problems.

[0008] The present invention also provides embodiments of the above-mentioned carrier, in which the non-chargeable segment is polyethylene glycol; the chargeable segment is polyamino acid and the block copolymer is shown by any of the following formula (I) and (II);



(where,  $R_1$  is a hydrogen atom, a hydrocarbon group or a functional group or a functional group substituted hydrocarbon group;  $R_2$  is NH, CO or  $R_6(CH_2)_qR_7$ , where  $R_6$  indicates OCO, OCONH, NHCO, NHCOO, NHCONH, CONH or COO,  $R_7$  indicates NH or CO, and q indicates an integer of 1 or more;  $R_3$  is a carboxyl group, a carboxyl group substituted hydrocarbon group, an amino group substituted hydrocarbon group, a hydrazino group, substituted hydrocarbon group,  $(CH_2)_p-NHCNHNH_2$  group, where p

indicates an integer of 1 or more, a nitrogen-containing heterocyclic group or nitrogen-containing heterocyclic group substituted hydrocarbon group; R<sub>4</sub> is a hydrogen atom, a hydroxyl group or hydrocarbon group having any of CO, NH and O at the bonding terminal thereof; m is a number within a range of from 4 to 2,500; n is a number within a range of from 1 to 300; and x is a number within a range of from 0 to 300, provided that x < n).

[0009] In addition, the present invention provides an electrostatic bonding macromolecular micelle carrier drug in which a drug is carried by the carrier as described above, and a method for the manufacture thereof.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Fig. 1 shows a spectral chart of 1H-NMR of PEG-P(Lys).

[0011] Fig. 2 shows a graph comparing measuring results of melting for cases with PEG-P(Lys)/DNA, free DNA and (Lys)/DNA.

#### DETAILED DESCRIPTION OF THE INVENTION

[0012] The present invention as described above was developed as a result of studies carried out by the present inventors to overcome the problems in the conventional physical adsorption type macromolecular micelle drug, and realizes a novel electrostatic bonding type macromolecular micelle drug carrier essentially different from the physical adsorption type one, drugs carried by means thereof, and a method for carrying the drug.

[0013] In the electrostatic bonding macromolecular micelle carrier comprising a non-chargeable segment and a chargeable segment of the present-invention as described above, various substances are applicable for the both segments within the scope of the present invention.

[0014] Applicable non-chargeable segments include, for example, polyalkylene glycol such as polyethylene glycol and polypropylene glycol, polyalkylene oxide, polysaccharide, polyacrylamide, poly-substituted acrylamide, polymethacrylamide, poly-substituted

methacrylamide, polyvinylpyrrolidone, polyvinyl alcohol, polyacrylic acid ester, polymethacrylic acid ester, polyamino acid, and derivatives thereof.

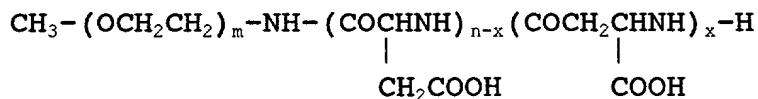
[0015] Applicable chargeable segments include, for example, a polyamino acid having a chargeable side chain, or more specifically, polyaspartic acid, polyglutamic acid, polylysine, polyarginine, polyhistidine, or, polymalic acid, polyacrylic acid, polymethacrylic acid, polyethylene imine, polyvinylamine, polyacrylamine, polyvinyl imidazole, and derivatives thereof.

[0016] Substances applicable as a block copolymer of the present invention comprising these segments include;

[0017] Polyethylene glycol-polyaspartic acid block copolymer, polyethylene oxide-polyglutamic acid block copolymer, polyethylene glycol-polyarginine block copolymer, polyethylene glycol-polyhistidine block copolymer, polyethylene glycol-poly-methacrylic acid block copolymer, polyethylene-polyvinylamine block copolymer, polyethylene glycol-polyarylamine block copolymer, polyethylene oxide-polyaspartic acid block copolymer, polyethylene oxide-polyglutamic acid block copolymer, polyethylene oxide-polylysine block copolymer, polyethylene oxide-polyacrylic acid copolymer, polyethylene oxide-polyvinyl imidazole block copolymer, polyacrylamide-polyaspartic acid block copolymer, polyacrylamide-polyhistidine block copolymer, polymethacrylamide-polyacrylic acid block copolymer, polymethacrylamide-polyvinylamine block copolymer, polyvinylpyrrolidone-polyaspartic acid block copolymer, polyvinylalcohol-polyarginine block copolymer, polyacrylic acid ester-polyhistidine block copolymer, polymethacrylic acid ester-polyvinylamine block copolymer, and polymethacrylic acid-polyvinylimidazole block copolymer.

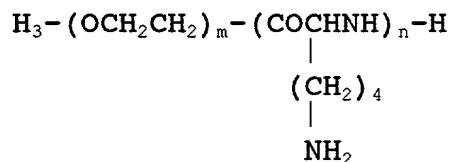
[0018] A representative structure of these block copolymers is one known as AB-type block copolymer.

[0019] More specifically, the following paragraph describes an AB-type block copolymer comprising a non-chargeable segment obtained from a polyethylene glycol derivative and polyaspartic acid as the chargeable segment;



[0020] This is a polyethylene glycol-poly( $\alpha$ ,  $\beta$ -aspartic acid) block copolymer comprising polyethylene glycol and poly( $\alpha$ ,  $\beta$ -aspartic acid), and is synthesized by copolymerizing  $\beta$ -benzyl-L-aspartate-N-carboxylic anhydride with poly-ethylene glycol which is a unilateral terminal amino group (molecular weight: 200 to 250,000) as the initiating agent. The molecular weight of the ( $\beta$ -benzyl, L-aspartate) portion of this polyethylene glycol ( $\beta$ -benzyl-L-aspartate) block copolymer is variable within a range of from about 205 to 62,000. Polyethylene glycol-poly( $\alpha$ ,  $\beta$ -aspartic acid) block copolymer is available by eliminating benzyl through application of an alkali treatment of this copolymer.

[0021] Polyethylene glycol-polylysine block copolymer, shown by the following formula, having a cationic segment as the block copolymer:



is synthesized through polymerization of  $\epsilon$ -carbobenzoxy-L-lysine anhydride with unilateral terminal primary amino group polyethylene glycol (molecular weight: 200 to 250,000) as the initiating agent. Polyethylene glycol-polylysine block copolymer is available by subjecting the resultant polyethylene glycol-poly( $\epsilon$ -carbobenzoxy-L-lysine) block copolymer to a deprotecting reaction by the use of methane sulfonic acid.

[0022] In the present invention, while there is no particular limitation in the kind of drugs capable of being electrostatically carried in a macromolecular micelle comprising a block copolymer as described above, applicable ones include macromolecular drugs such as peptide hormones, proteins, DNA, RNA, and oligonucleotides and low molecular weight drugs having a chargeable functional group the in molecule such as Adriamycin and Daranomycin.

[0023] When causing the macromolecular micelle to carry any of these drugs, it is the basic practice to mix the block copolymer and the drug or a solution thereof. Various operations including dialysis, stirring, dilution, concentration, ultrasonication, temperature control, pH control and addition of an organic solvent may appropriately be adapted.

[0024] When including lyoszyme, an antimicrobial enzyme, in the polyethylene glycol-poly( $\alpha$ ,  $\beta$ -aspartic acid) block copolymer shown above, lyoszyme can be carried by mixing an aqueous solution of the copolymer with an aqueous solution of lyoszyme under appropriate conditions including mixing ratio, ionic strength and pH.

[0025] Furthermore, when causing the polyethylene glycol polylysine block copolymer described above to carry DNA, it is possible to conduct the DNA to be carried by mixing an aqueous solution of the copolymer with an aqueous DNA solution under conditions including appropriate mixing ratio, ionic strength and pH.

[0026] As described above, according to the electrostatic bonding macromolecular micelle drug carrier and the carried drug using same of the present invention, a stable macromolecular micelle structure is available and chargeable substances such as protein and DNA can be efficiently incorporated into the internal nucleus thereof. It is thus decomposed in vivo into the body in a stable state.

[0027] The present invention is now described further in detail by means of examples. It is needless to mention that the present invention is not limited to these examples.

[0028] Example 1

Poly-L-lysine (degree of polymerization: 20,043 mg) was dissolved into distilled water (1.0ml), and a polyethylene glycol-polyaspartic acid block copolymer (PEG-P(Asp): molecular weight of PEG: 5,000, 23 aspartic acid residues per a chain of the block copolymer, 1.0 mg) was dissolved into distilled water (1.0 ml). Thereafter, these aqueous solutions were mixed. A weight average particle size of 41.3 nm and a number average particle size of 36.0 nm of the resultant mixture were measured by the method of dynamic light scattering. A zeta-potential of 0.643 and 0.569 mV for the entire surface of the mixture was measured by the method of trophoretic light scattering.

**[0029] Example 2**

Polyaspartic acid (degree of polymerization: 20, 0.32 mg) was dissolved into distilled water (1.0 ml), and polyethylene glycol-poly-L-lysine block copolymer PEG-P(Lys); molecular weight of PEG: 5,000, 20 L-lysine residues per chain of block copolymer, 1.0 mg) was dissolved into distilled water (1.0 ml). Thereafter, these aqueous solutions were mixed. A weight average particle size of 28.2 nm and a number average particle size of 42.8 nm of the resultant mixture were measured by the method of dynamic light scattering.

**[0030] Example 3**

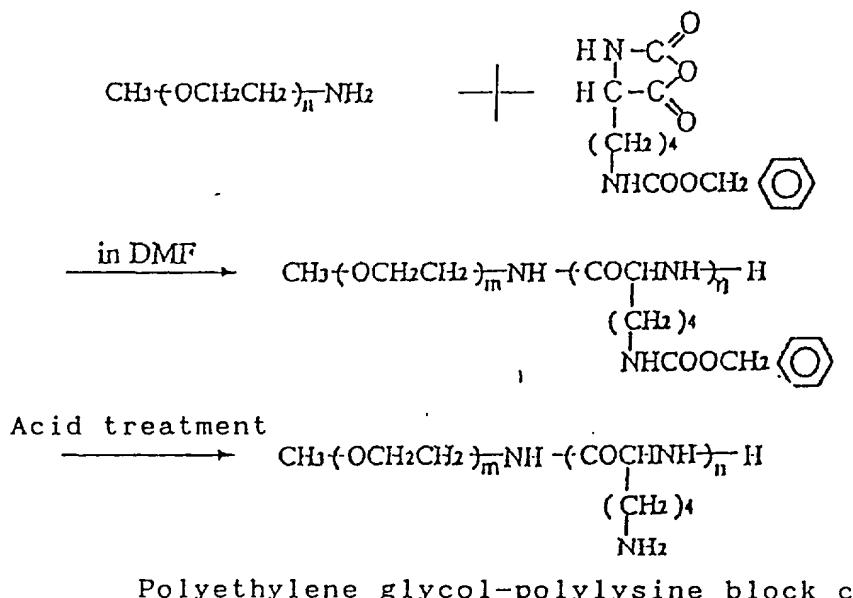
Chicken albumen lysozyme (1.0 mg) was dissolved into distilled water (1.0 ml), and PEG-P(Asp) (3.0 mg) was dissolved into distilled water (3.0 ml). Thereafter, these solutions were mixed. A weight average particle size of 24.9 nm and a number average particle size of 23.1 nm of the resultant mixture were measured by the method of dynamic light scattering.

**[0031] Example 4**

Bovine insulin (1.42 mg) was dissolved into a 0.0005N hydrochloric acid (1.5 ml), and PEG-P(Lys) having a particle size of 0.58 mg was dissolved into distilled water (1.0 ml). Thereafter, these solutions were mixed. A weight average particle size of 24.5 nm, and a number average particle size of 22.4 nm of the mixed solution were measured by the method of dynamic light scattering.

**[0032] Example 5**

A polyethylene glycol-polylysine block copolymer was synthesized in accordance with the following formula:



[0033] Fig. 1 shows <sup>1</sup>H-NMR spectra for a case with a PEG molecular weight of 4,300 and 20 L-lysine residues.

[0034] This PEG-P(Lys) block copolymer (PEG molecular weight; 4,300, average degree of polymerization of polylysine chain; 20) was dissolved into 1.0 ml of 0.1 M PBS (pH: 7.4) solution of Salmon Testes DNA in an amount of 50 ug/ml, and into 1.0 ml of 0.1 M PBS + 0.6 M NaCl + 2mM Na<sub>2</sub>EDTA (pH: 7.4) so that the number of lysine residues of PEG-P(Lys) relative to DNA phosphate group became 0.25, 0.50, 1.0, 2.0, 4.0, 10 and 20 times as large, respectively. These solutions were mixed and then held at the room temperature for three hours. No precipitation was observed in any of these samples. For a complex using polylysine homopolymer, on the other hand, precipitation took place in samples with ratios (=r) of lysine residues: DNA phosphate group of 1.0 and 2.0. Subsequently, a 20 μ l fraction was taken from each sample and subjected to electrophoresis using 0.9% agarose gel. As a result, the amount of DNA migrating along with the increase in the amount of PEG-P(Lys) added to DNA decreased, and DNA migration was almost inhibited at an amount of addition (r = 1.0) of PEG-P(Lys) with which the charge became equivalent to that of DNA. It was consequently confirmed that a quantitatively stable poly ion complex was formed by the PEG-P(Lys) block copolymer and DNA.

[0035] When using a polylysine homopolymer (molecular weight: 1,000 to 4,000) having a degree of polymerization almost equal to that of the PEG-P(Lys) block copolymer, inhibition of DNA migration by addition of polylysine homopolymer was not observed and a stable complex was unavailable.

[0036] Example 6

A PEG-P(Lys) block copolymer was dissolved into 1.0 ml of 1mM PBS (pH: 7.4) solution of Salmon Testes DNA in an amount of  $50 \mu \text{g/ml}$ , and into 1.0ml of 1mM PBS (pH: 7.4) so that the number of lysine residues of PEG-P(Lys) relative to DNA phosphate group became 0.10, 0.20, 0.50 and 1.0 times as large, respectively. A complex was formed by mixing these solutions. After holding the complex at  $4^\circ\text{C}$  for a night, the thermal melting curve of each sample was measured by adding methanol in an amount of 50 vol.% by the use of ultraviolet absorption ay 260 nm.

[0037] As a result, while the control DNA showed a first melting stage at about  $45^\circ\text{C}$ , the complex of DNA and PEG-P(Llys) showed two stages of melting at about  $45^\circ\text{C}$  and about  $65^\circ\text{C}$ . The increase in absorbance at about  $45^\circ\text{C}$  gradually decreased according as the amount of added PEG-P(Lys) was increased, whereas the increment of absorbance at about  $65^\circ\text{C}$  in that place. In the sample in which PEG-P(Lys) was added up to 1.0 times to DNA, the increase in absorbance at about  $45^\circ\text{C}$  disappears, and only the increase in absorbance at about  $65^\circ\text{C}$  was observed, suggesting that the structure of DNA was completely stabilized. This confirmed that DNA and PEG-P(Lys) stoichiometrically form a complex.

[0038] Fig. 2 shows a case where the number of lysine residues of PEG-P(Lys) is equal to 0.50 times relative to DNA phosphate group, and cases with free DNA and P(Lys)/DNA.

[0039] Remarkable differences are observed also in Fig. 2.

**[0040] Example 7**

Poly-L-lysine (degree of polymerization: 20) (40 mg) was dissolved into 4 ml of the phosphate buffer solution, and polyethylene glycol-polyaspartic acid block copolymer(PEG-P(Asp); molecular weight of PEG: 5000, 20 aspartic acid residues per a chain of the block copolymer, 2, 32mg) was dissolved into 2.32 ml of the phosphate buffer solution.

**[0041]** Thereafter, these aqueous solutions were mixed. A weight average particle size of 44.7 nm and a number average particle size of 41.3 nm of the resultant mixture were measured by the method of dynamic light scattering.

**[0042] Example 8**

Poly-L-lysine (degree of polymerization:20) was dissolved into 4 ml of the phosphate buffer solution, and PEG-P(Asp) (molecular weight of PEG:5000, 80 aspartic acid residues per a chain of the block copolymer 4.5mg) was dissolved into 4.5 ml of the phosphate buffer solution. Therefore, these aqueous solution were mixed. A weight average particle size of 43.6 nm and a number average particle size of 41.8 nm of the resultant mixture are measured by the method of dynamic light scattering.

**[0043] Example 9**

Polyethylene glycol-poly-L-lysine block copolymer(PEG-PLys:(molecular weight of PEG:500, 20 lysine residues per a chain of the block copolymer, 5mg) was dissolved into 1ml of the phosphate buffer solution, and polyethylene glycol-polyaspartic acid block copolymer(PEG-P(Asp): molecular weight of PEG: 5000, 20 aspartic acid residues per a chain of the block copolymer, 5mg, was dissolved into 1 ml of the phosphate buffer solution.

**[0044]** Thereafter, these aqueous solutions were mixed. A weight average particle size of 30.8 nm and a number average particle size of 28.8 nm of the resultant mixture were measured by the method of dynamic light scattering.

**[0045]** According to the present invention, as described above in detail, there are provided a carrier capable of stably carrying a drug under the effect of a macromolecular micelle structure, and a drug carried by this carrier. It is possible to stably incorporate chargeable substances such as protein and DNA which tend to be easily decomposed in vivo.